Mechanism of Microbiological Contamination of Jet Fuel and Development of Techniques for Detection of Microbiological Contamination

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FOREWORD

This is the fifth quarterly progress report prepared under Contract AF 33(657)-9186, "Mechanism of Microbiological Contamination of Jet Fuels and Development of Techniques for Detection of Microbiological Contamination." This contract was initiated by the AF Aero Propulsion Laboratory, Research and Technology Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Mr. Jack Fultz is the Project Engineer.

This report concerns work done from 1 June 1964 to 1 September 1964.

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 Since this is a progress report, the information herein is tentative and subject to changes, corrections, and modifications.

ABSTRACT

This study is concerned with the detection of microorganisms in fuel-water environments and with the mechanisms by which these microorganisms cause problems in fuel systems of aircraft. Fuel isolates removed nitrate from growth medium containing iron and calcium, causing it to become more corrosive to aluminum alloys. Upon prolonged incubation (200 days) fuel isolates produced substances corrosive to aluminum alloys. Microbial sludge, which can cause corrosion, contained N, C, H, and O. Nitrate did not prevent corrosion caused by nitrated phenols; and nitrate does not prevent corrosion caused by media in which fuel isolates have grown for long periods of time. Emulsion-forming organisms contained considerably more lipid material than fuel isolates which were sedimentable in water. Some fuel additives were observed to support the growth of fuel isolates while most anti-icing and metal deactivators were mildly toxic. Respiratory inhibition and ability to kill fuel isolates was demonstrated for pentene, hexene, heptene, octene, and nonene but not for decene or dodecene. The saturated homologs of these compounds were either innocuous or supported growth.

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1. INTRODUCTION

The major objective of this program is to determine how fuel contaminants such as water, surface-active substances, extraneous solid materials, and rust, singly or in combination, react to cause problems in jet fuel systems. The study is directed at the effect of these fuel system contaminants on microbial growth and metabolism, as well as the reciprocal effect of microbial growth on the ability of contaminants to cause difficulties in jet fuel systems.

Some of the mechanisms by which microorganisms cause corrosion in fuel-water systems have been elucidated. Past efforts have been directed to an investigation of the concept that microorganisms remove mineral inhibitors from media in which they grow and thereby stimulate metal corrosion. The operation of this mechanism in fuel isolates with respect to iron and calcium corrosion stimulation, and nitrate and phosphate inhibition has been demonstrated.

In this period, greater emphasis has been given to the investigation of possible corrosive compounds produced by microorganisms which have grown in mineral media on jet fuel for long periods of time. This research has yielded evidence for corrosive compounds produced both in media with initially high and low concentrations of nitrate. Microbial sludges from low-nitrate media caused corrosion, and water-soluble compounds from high-nitrate media caused corrosion. The latter corrosion was not inhibited by nitrate.

Other research concerned with the investigation of the metabolic pathways operative in fuel oxidation by microbial contaminants was continued; and research was accomplished on the biosynthesis of compounds from fuel hydrocarbons. Two kinds of microbial products were analyzed chemically, both of which appear capable of interfering with fuel system operation and both of which derive from fuel oxidation. One product is lipid in character and contributes to microbial emulsion formation. The other product is similar to a wax and is insoluble both in water and jet fuel. The ability of certain substances in sealants and topcoats to sustain the growth of organisms found in fuel-water systems was investigated, and the effect of short-chain hydrocarbons and olefins on fuel oxidation was measured. Attention was given to exploring the effect of fuel additives on microbial growth in fuel-water systems. The ability of such additives to prevent aluminum corrosion caused by biologically essential ions was studied.

II. SUMMARY AND CONCLUSIONS

As a tentative conceptual structure in planning research on microbial contamination of fuel, four hypotheses were proposed by which microorganisms could bring about aluminum corrosion. It appeared probable that each of the corrosion mechanisms played some part in causing aluminum corrosion by microorganisms.

Past studies have been concerned with investigating the validity of the first hypothesis; i.e., that microorganisms cause corrosion by altering the relative concentration of biologically essential ions in a growth medium. The results of this effort showed that the removal, by bacteria, of phosphate and nitrate from a growth-supporting medium caused the medium to become more corrosive to aluminum. It was concluded that the greater corrosivity resulted from increasing the proportion of iron and calcium present. It was stressed that media supporting the growth and multiplication of microorganisms were intrinsically corrosive and that microorganisms can remove corrosion inhibitors such as nitrates or phosphate and cause the medium to become actively corrosive.

A second hypothesis was proposed and tested; i.e., that microorganisms can produce corrosive materials from the oxidation and transformation of hydrocarbon substrates. The actual production of such corrosive material was suggested by the corrosion of aluminum caused by microbial growth in a casein hydrolysate medium. This corrosion was not prevented by nitrate and appeared not to be stimulated by mineral constituents such as iron or calcium.

During the period of research reported on here, further work was performed on the production of corrosive compounds by fuel isolates. It was concluded that organisms did produce corrosive compounds when grown in mineral media for long periods of time. The time required for the production of these compounds was sometimes as great as 200 days. Corrosive insoluble sludge was produced by cultures initially low in nitrate; and soluble corrosive compounds were produced in cultures initially high in nitrate. Corrosion caused by the latter compounds was not prevented by the addition of nitrate.

Research was accomplished on a third hypothesis; i.e., that corrosion of aluminum is caused by microorganisms establishing microcenters of galvanic activity on metal surfaces. During this period it was shown that pitting corrosion took place under coating holidays. These coating lesions were produced by degenerative changes in microbial culture media. It was concluded that local galvanic activity contributed to the local pitting observed.

Attempts were made to determine the contribution of the mechanism described in a fourth hypothesis to the corrosion phenomena observed. It was theorized that microorganisms remove electrons from the metal surface,

but the demonstration of the direct oxidation and corrosion of aluminum by microorganisms has been unsuccessful. However, when electron mediators were added to microbial cultures they caused the deposition of microbial material or metabolic products on aluminum surfaces. The conclusion was drawn, therefore, that these agents brought about conditions which led to aluminum corrosion.

Chemical analyses were made of insoluble sludge produced by microorganisms and the material was found to contain nitrogen. Experiments demonstrated that nitrated hydrocarbons stimulated corrosion of aluminum and that this stimulation was not prevented by inorganic nitrate. It was concluded that nitrate did not prevent the corrosion caused by soluble material in exhausted cultures which were at one time rich in nitrate.

The effect of common fuel additives on the growth and survival of fuel isolates and on corrosion was studied. Some additives actively stimulated microbial growth while others were mildly toxic at high concentrations.

Both microbial sludge and emulsions were analyzed chemically and found to contain respectively C, H, N, and O, as well as fatty acids. The C, H, N, and O could produce corrosion and the fatty acids could cause emulsion formation.

Research on the metabolism of fuel isolates showed that the toxicity of short-chain olefins ended with the ten carbon mono-unsaturated hydrocarbons. A general parallel was established between the effect of short-chain unsaturated hydrocarbons on viability and on respiratory inhibition.

VII. FUTURE WORK PLANNED

Work planned for the next quarter will deal with the chemical composition of sludges and emulsions produced by fuel organisms and with the characteristics of the metabolic pathways involved in their production. Environmental conditions which possibly alter these pathways will be studied. Whole cells and cell-free extracts will be used in the investigation of hydrocarbon oxidation.

The acids produced by microbial fuel oxidation will be studied. Attempts will be made to concentrate and purify these materials. If some degree of purification is achieved, tests will be made of the effect of these acids on aluminum alloy corrosion, and on their emulsifying properties.

The ability of microbial emulsions and microbial sludge to concentrate corrosive minerals will be determined.

Further efforts will be made to chemically distinguish microbial corrosion products on the aluminum from those produced by minerals.

Work on the effect of fuel additives on the metabolism of fuel organisms will be continued.

IV. RESULTS AND DISCUSSION

The work for this phase has been concerned with the mechanism of corrosion, hydrocarbon oxidation, sludge formation, sealant and topcoat deterioration, and the role of additives in each of these processes. Each of these tasks is described in detail below.

A. Mechanism of Aluminum Alloy Corrosion by Microorganisms

1. Alteration in Biologically Essential Ions

a. Nitrate Utilization: The utilization of nitrate for the growth of microorganisms may involve its reduction to nitrite and ammonia, while microorganisms in media containing nitrate also may effect the reduction of this compound to gaseous nitrogen. It was of interest, therefore, to determine the residual concentration of nitrate and nitrite in microbial cultures which contained initially 1.2 g KNO_3 per liter and which had contained microbial growth for about 3 months. This interest stems from the fact that aluminum coupons submerged in these cultures were extensively corroded only after 97 days. The medium inhibits corrosion at the beginning of growth because of the large concentration of nitrate present.

Nitrate and nitrite were determined respectively by the methods of Skujins[1] and Pappenhagen and Mellon.[2] Using these methods, analysis revealed that cultures which contained initially 1.2×10^{-2} moles of NO_3^- contained only 10^{-4} moles after 89 days incubation. (See Table 1.) These analyses revealed also that part of the nitrate acted upon by the organisms was reduced to nitrite. Thus, cultures containing 1.2×10^{-2} moles KNO_3 initially, and no added nitrite, had 2×10^{-3} moles NO_2^- present per liter after 89 days of growth.

Data on Table 1 shows that in a medium with 1.2 g KNO_3 per liter initially, the nitrate concentration decreased by a factor of 126.5 in the first 19 days whereas the nitrite concentration increased from 0 to 2.07×10^{-3} moles per liter. After this, the concentrations of both ions were essentially unchanged. But corrosion was only apparent after 66 days of exposure, while media with 0.02 g KNO_3 per liter initially produced aluminum corrosion within 20 days. It would appear that between the 19th day of incubation and the 66th day of incubation, certain processes had occurred which made the medium corrosive.[3] This increase in corrosivity appeared to be dissimilar to that observed in media with low nitrate concentrations. As pointed out above, 0.02 g KNO_3 per liter media with the same concentration of calcium and iron as media with a higher nitrate concentration caused corrosion in only about 20 days. In media at high initial nitrate concentrations a longer period of time was required for the appearance of corrosion, even when the medium has been essentially depleted of nitrate by microbial growth.

TABLE 1

NITRATE REDUCTION BY FUEL ISOLATES IN MEDIUM CONTAINING HIGH CONCENTRATIONS OF KNO_3

Time (Day)	Nitrate (Moles/Liter)	Nitrite (Moles/Liter)
0	120.0×10^{-4}	0
19	0.949×10^{-4}	20.7×10^{-4}
43	1.19×10^{-4}	19.5×10^{-4}
89	0.791×10^{-4}	20.7×10^{-4}

Legend: Bushnell-Haas fuel medium containing 1.2 grams KNO_3 as the only nitrogen source was inoculated with fuel-grown mixed culture which had been washed 3 times in distilled water. The culture was placed on a rotary shaker at 30°C and nitrate determination by the method of Skujins[1] and nitrate determinations by the method of Pappenhagen and Mellon[2] were made at the indicated time intervals. Initial microscopic count of the mixed inoculum 10^7. After 89 days the microscopic count was 10^9.

These data would suggest that the absence of the aluminum corrosion inhibitor, nitrate, could have brought about or permitted the extensive corrosion of aluminum alloys observed in these cultures. But nitrite is a known corrosion inhibitor, and the accumulation of this compound in the culture medium implies that a corrosive substance had been elaborated in quantities that overcame the inhibitory effect of the nitrite ion.

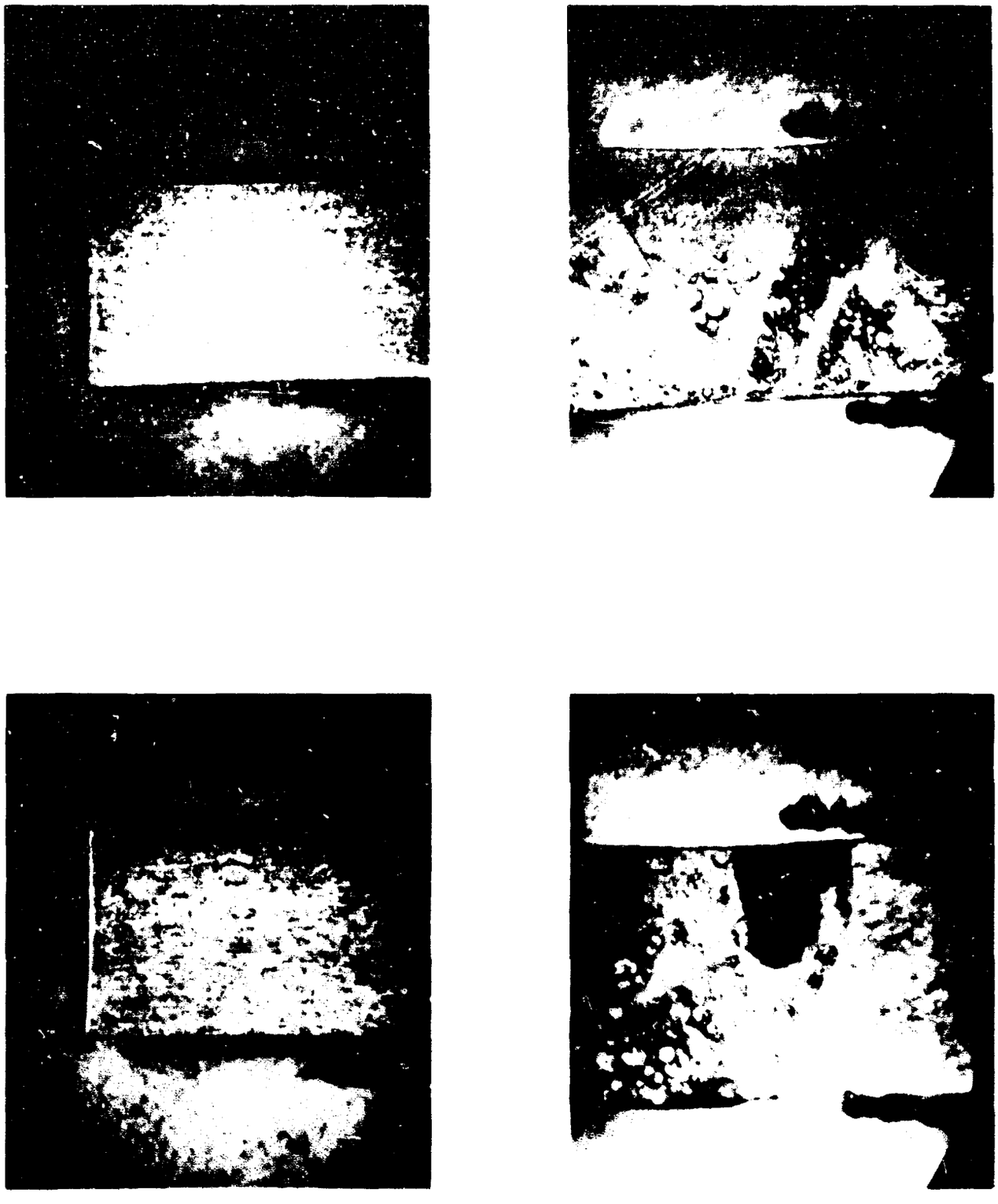
2. Production of Corrosive Compounds

a. Water-Soluble Compounds: It was believed that the increased corrosivity of the medium high in nitrate, described above, resulted from the microbial production of a corrosive compound. (See Table 1.) To test this assumption, modified Bushnell-Haas medium was prepared which contained 1.2 g KNO_3 per liter, and this medium was inoculated with a mixed culture of microorganisms isolated from jet fuel systems. The cultures were placed on a rotary shaker and incubated for 86 days. At the end of this period coupons of aluminum alloys 2024 and 7075 were immersed in the medium and inspected after 48 hours. Figure 1 shows that both alloys were corroded severely by these media by the end of this brief incubation period, but coupons in sterile media did not corrode. The corrosion occurring in these exhausted media was attributed to the presence of ferric hydroxide and calcium ion and to the absence of nitrate.

Other tests were run to better define the relationship of the advent of corrosion and nitrate depletion in these cultures. Accordingly 1.2 g KNO_3 was added to those exhausted cultures which had previously contained this concentration of nitrate. Aluminum coupons of alloys 2024 and 7075 were submerged in these media and inspected at the end of 48 hours. Figure 2 shows the corrosion which took place on the two alloys in media to which nitrate was added for the second time. Nitrate did not inhibit the corrosion stimulated by the medium in which microorganisms had grown for protracted time periods. It is to be observed that the uninoculated medium was not itself corrosive.

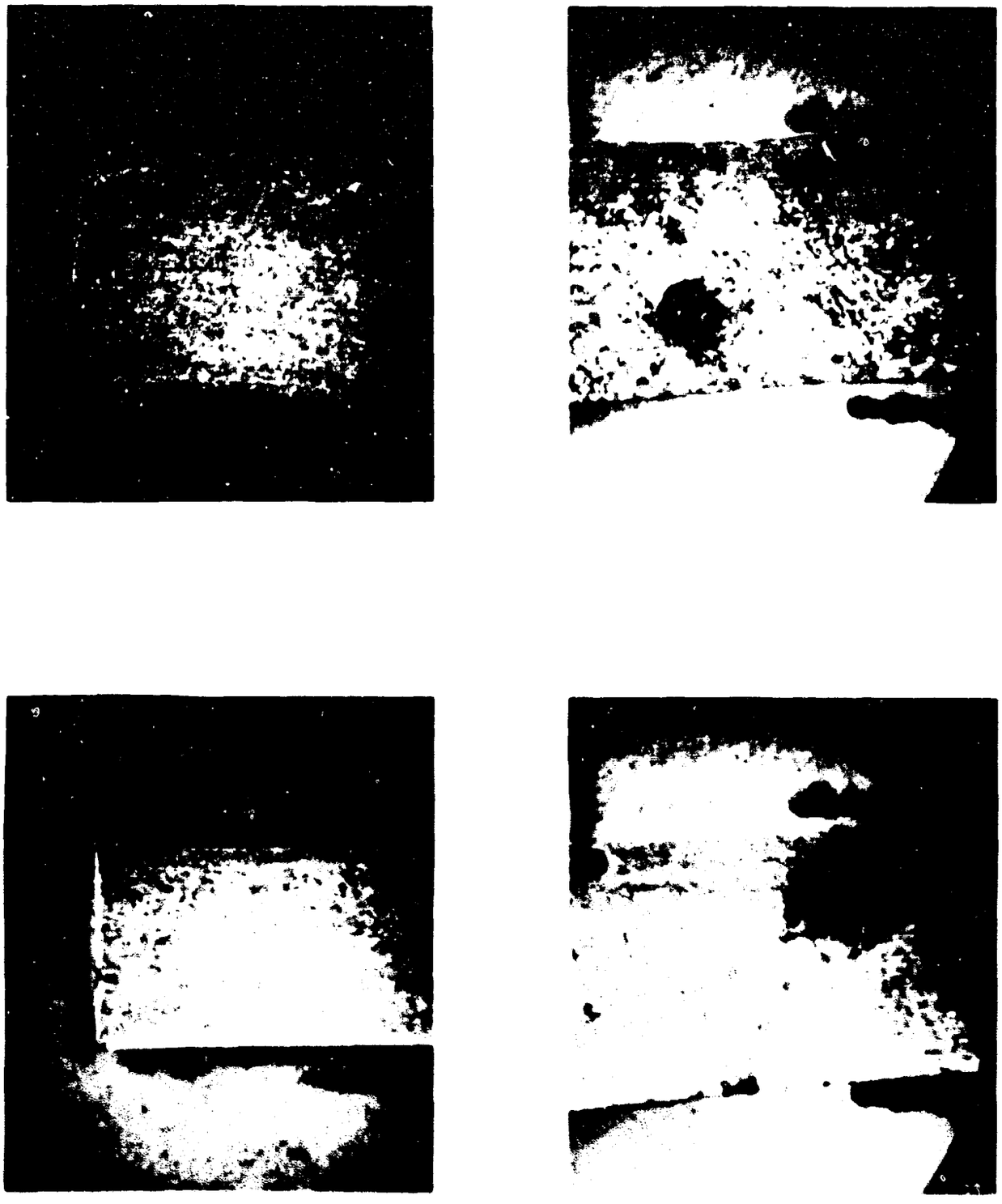
These results suggest that compounds are produced by fuel isolates which cause corrosion independently of the corrosion which may be stimulated by the presence of cations in the medium, such as iron and calcium. The corrosive action of these compounds appears to take place in the presence of inhibitors such as nitrate and phosphate.

b. Water-Insoluble Compounds (Sludge): Long-term microbial corrosion tests have been continued throughout the course of this investigation. One such test was set up to examine the possible sequential changes in compounds produced from fuels by microorganisms. As reported previously, this investigation revealed that cultures which contain high nitrate concentrations produce fuel-soluble compounds and microbial sludges.[3] The production of this sludge, which imparts a dramatically different appearance to the culture, has



LEGEND. FLASKS OF NBH MEDIUM WITH 1.2 g KNO_3 WERE INOCULATED WITH A MIXED CULTURE OF FUEL ISOLATES AND INCUBATED AT 30°C FOR 86 DAYS. ALLOYS 7075 AND 2024 WERE THEN ADDED AND THE FLASKS WERE INCUBATED FOR 48 HOURS. CONTROL BARS OF ALLOY 2024 AND 7075 WHICH WERE NOT IMMERSED IN CORROSIVE MEDIUM ARE PRESENTED ON THE LEFT. 7075 ALLOYS ARE IN THE BOTTOM ROW AND 2024 ALLOYS IN THE TOP ROW.

Figure 1. Corrosivity of Medium Following Long Term Microbial Growth



LEGEND. FLASKS OF NBH MEDIUM WITH 1.2 gm KNO_3 WERE INOCULATED WITH A MIXED CULTURE OF FUEL ISOLATES AND INCUBATED AT 30°C FOR 86 DAYS. ALLOYS 7075 AND 2024, AND A SECOND QUANTITY OF KNO_3 (1.2 GRAMS PER LITER) WERE THEN ADDED AND THE FLASKS WERE INCUBATED FOR 48 HOURS. CONTROL BARS OF EACH ALLOY WHICH WERE NOT IMMERSED IN CORROSIVE MEDIUM ARE PRESENTED ON THE LEFT. 7075 ALLOYS ARE PRESENTED IN THE BOTTOM ROW AND 2024 ALLOYS IN THE TOP ROW.

Figure 2. The Effect of Second Addition KNO_3 on the Corrosivity of Medium Following Long Term Microbial Growth

required as much as 200 days of incubation in modified Bushnell-Haas media maintained continuously with a JP-4 jet fuel overlay. Sterile media maintained under the same conditions do not produce sludge.

It was of interest to test the corrosivity of sludge formed in media containing 0.02, 0.06, 0.08 and 1.2 g KNO_3 per liter while the other constituants of the BH medium were maintained as published.[3] Sludge was collected from the bottoms of fifteen replica cultures at each of the concentrations of nitrate indicated above. The sludge from cultures of a given nitrate concentration were combined in a centrifuge tube and washed three times with 100 volumes in water at each washing. The sludge was violently agitated for 15 minutes between washings to effect possible equilibration of entrapped ions with the water phase.

Following the cleaning of the sludge, aluminum coupons of 2024 and 7075 were submerged in each sludge sample and examined periodically. Figure 3 shows the pattern of corrosion observed in the surface of the two alloys tested. The sludge from the culture containing the least nitrate, 0.02 g per liter, and yielding the least quantity of sludge was by far the most corrosive product. While Figure 3 does not well illustrate the point, the extent of corrosion on coupons immersed in the sludge suspension diminished with sludges obtained from cultures which had grown on progressively higher nitrate concentrations. This pattern of corrosion may prove to be the converse of that observed with the supernatants of very old cultures. As indicated in Figure 1, the supernatant which yielded noncorrosive sludge was itself corrosive.

It is believed that obtaining a microbial product which can cause corrosion is of value to this study because it permits an analysis of the reactions with aluminum surfaces of natural organic products formed by microorganisms. The analysis of the sludge itself will give clues to the metabolic processes involved in its synthesis, and the metabolic processes operative in fuel-oxidizing organisms in general.

c. Effect of Nitrate on Aluminum Corrosion Caused by Nitrated Hydrocarbons: Previous studies demonstrated that the pseudomonads isolated from jet fuel systems were especially resistant to the bacteriostatic action of 2, 4-dinitrophenol (DNP), and, furthermore, this nitrated phenol was a powerful stimulant of aluminum alloy corrosion.

The action of DNP as a corrosion stimulant became of interest again in this period with the realization that microbial sludges formed at high nitrate concentrations were nitrogen-containing compounds. Tests were set up to find the extent to which nitrate ions might inhibit aluminum corrosion caused by DNP. These tests show that corrosion took place on aluminum coupons in DNP solutions both in the presence and absence of equal molar concentrations of nitrate. This result is analogous to that obtained in exhausted media to which nitrate had been added (Figure 2). There, also,

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LEGEND SLUDGE WAS COLLECTED FROM MEDIA CONTAINING FROM LEFT TO RIGHT, 1.2, 0.08, 0.06, AND 0.02 GRAMS KNO_3 PER LITER AND WASHED THREE TIMES WITH DISTILLED WATER. ALLOYS 2024 AND 7075 AND JP-4 FUEL WERE ADDED TO EACH SAMPLE OF SLUDGE AND THE SAMPLES INCUBATED ON A ROTARY SHAKER 30°C FOR 48 HOURS. ALLOYS 7075 ARE SHOWN ON THE LEFT, 2024 ON THE RIGHT.

Figure 3. Corrosivity of Bacterial Sludge Formed in Media Containing Different Concentrations of KNO_3

nitrate appeared to be incapable of inhibiting the corrosion brought about by a compound of microbial origin. This parallel action suggests that, perhaps, a water-soluble microbial product such as that characterized by absorption spectrum and pK may be an active corrosion stimulant.[3] Microorganisms are capable of forming ring compounds containing nitrogen, and ring systems with nitrogen substituants. In such configurations, as well as in nitrate, the nitrogen may acquire corrosive properties.

3. Transfer of Electrons from Metal to Electron Acceptor

The possibility was investigated that microorganisms might produce substances which act as mediators in electron transfer from metal to oxygen or to some biological electron acceptor. In this system, methylene blue hydrochloride was used as a model electron transfer mediator. The function of methylene blue hydrochloride would be grossly similar to that of nickle in affecting aluminum oxidation and corrosion.

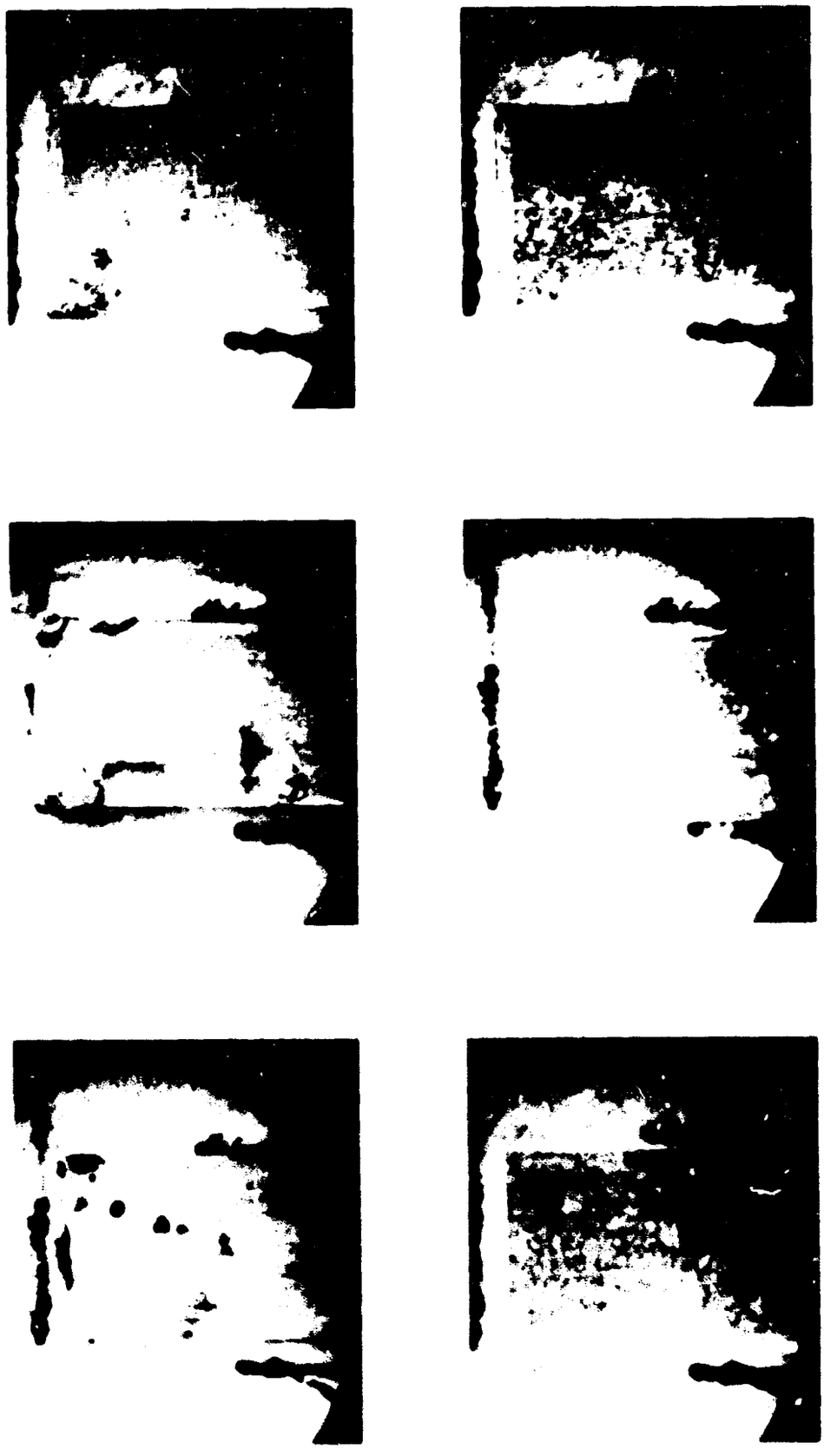
Cultures of fuel isolates were prepared in NBH with a JP-4 fuel overlay. Culture media were made to 8, 40, and 80 millimolar in methylene blue hydrochloride. The media were inoculated with fuel isolate culture 101, and coupons of aluminum alloys 2024 and 7075 were submerged in the aqueous phase of each culture. Controls contained these same concentrations of methylene blue and aluminum coupons but they were not inoculated with microorganisms.

By means of Figures 4 and 5 a comparison may be made of the effect of methylene blue on aluminum coupons in the presence and absence of microbial growth. The pictures were taken five days after inoculation. The cell concentration changed from 5×10^6 cells per ml to 3.2×10^9 cells per ml during this time. From Figure 4 it is apparent that the dye affects the aluminum surface very little in the absence of microbial growth; when this electron mediator and microorganisms are included in the same culture the organisms adhere to the surface of the aluminum. It was observed that pitting corrosion had occurred beneath the adsorbed organisms or debris. As stated, the duration of the experiment was 5 days; i.e., a time period in which cells of culture 101 did not precipitate on aluminum surfaces in the absence of methylene blue as observed in the past but not shown here. It is believed that the effect of methylene blue was predominately on the metabolic activity of the microorganisms rather than on surface of the aluminum coupon. These results emphasize again the necessity of carefully evaluating the environment and medium in which microorganisms cause corrosion, as well as the taxonomic classification of the organisms present.

B. Effects of Fuel Additives on Microorganisms and Corrosion

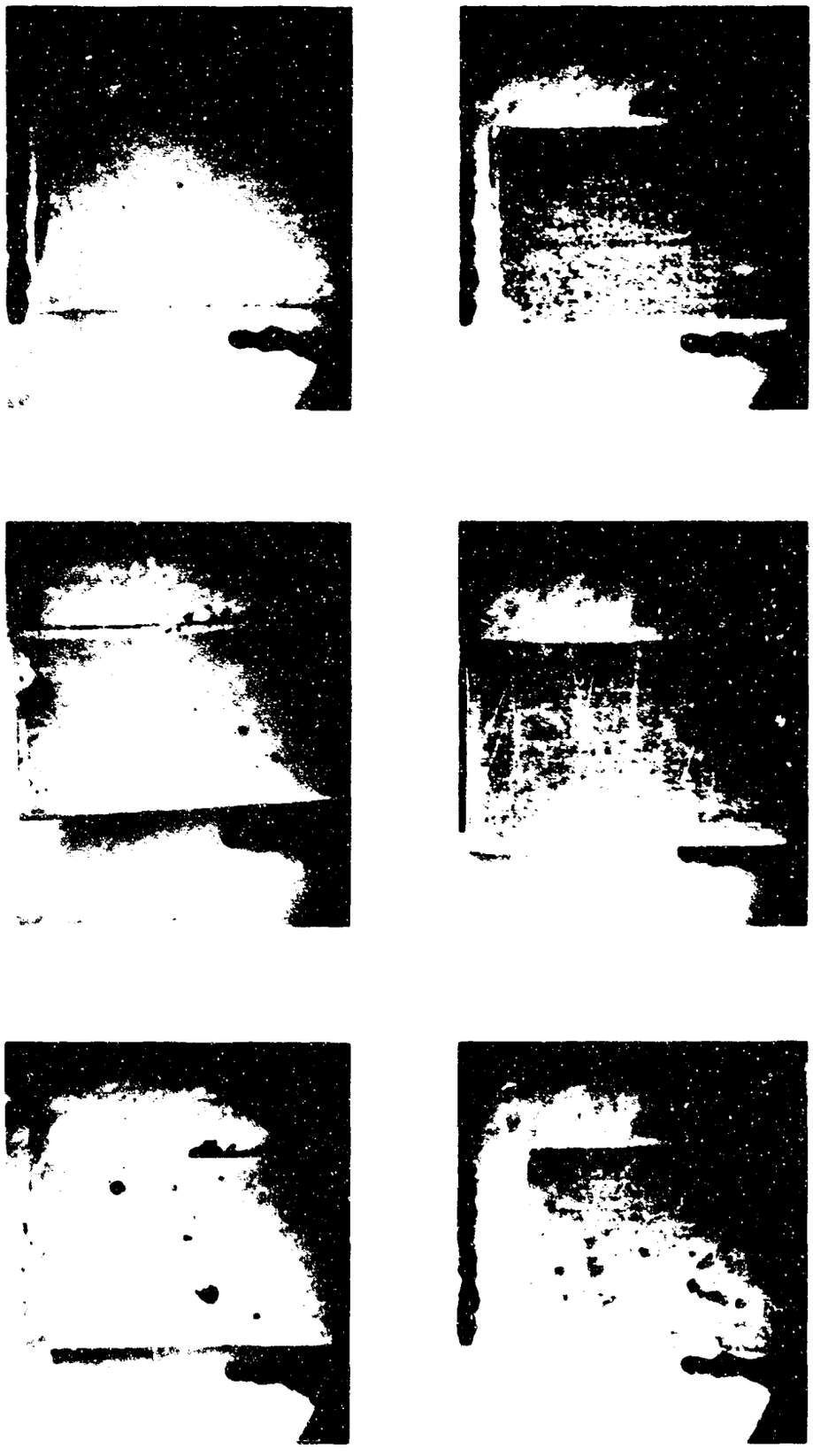
1. Effects on Growth and Survival of Microbes

Samples of antioxidants and anti-icing agents, currently approved for use in JP-4 jet fuel, were received by Melpar, Inc., from the Fuels and Lubricants Branch of the Technical Support Division of the Air Force Aero Propulsion Laboratory at Wright-Patterson Air Force Base, Ohio.



LEGEND THE NBH CULTURE MEDIUM WAS MADE 8 40 AND 80 mM IN MB. THE FLASKS WERE INOCULATED WITH CULTURE 101 AND ALLOY 7075 WAS ADDED TO EACH FLASK. CONTROL FLASKS PREPARED SIMULTANEOUSLY CONTAINED NO MICROORGANISMS. TESTS AND CONTROLS WERE INCUBATED 5 DAYS AT 30°C. CONTROLS ARE PRESENTED ON THE RIGHT, TESTS ON THE LEFT, FROM TOP TO BOTTOM MB CONCENTRATIONS IS 8 40 AND 80 mM RESPECTIVELY.

Figure 4. A Comparison of the Effect of Methylene Blue Hydrochloride (MB) on Aluminum Alloy 7075 in the Presence and Absence of Microbial Growth



LEGEND THE NBH CULTURE MEDIUM WAS UNDER 8, 40, AND 80 mM IN MB. THE FLASKS WERE INOCULATED WITH CULTURE 101 AND ALLOY 2024 WAS ADDED TO EACH FLASK. CONTROL FLASKS PREPARED SIMULTANEOUSLY CONTAINED NO MICROORGANISMS. TESTS AND CONTROLS WERE INCUBATED 5 DAYS AT 30°C. CONTROLS WERE PRESENTED ON THE RIGHT, TESTS ON THE LEFT. FROM TOP TO BOTTOM MB CONCENTRATION IS 8, 40, AND 80 mM RESPECTIVELY.

Figure 5. A Comparison of th Effect of Methylene Blue Hydrochloride (MB) on Aluminum Alloy 2024 in the Presence and Absence of Microbial Growth

Besides affecting microbial metabolism, antioxidants could alter the course of aluminum corrosion caused by microorganisms, and, also, either enhance, or diminish the rate of corrosion. Tests were made to determine the ability of organisms isolated from fuel to survive or grow in the presence of these compounds. Other long-term tests were set up to study the effect of antioxidants on the corrosion caused by large concentrations of microbes and on the aluminum corrosion caused by frequently encountered fuel contaminants such as rust.

For testing effects on microbial growth, media were prepared consisting of 100 ml of Bushnell-Haas medium with a 10 ml JP-4 overlay. To this fuel overlay from 0.05 ml to 10 ml of fuel additive was added. Viable cell counts were made periodically up to 24 hours.

If antioxidants are generally present in fuels, then it is probable that they affect the metabolism of fuel organisms and enter into corrosion and fuel system deterioration caused by other fuel contaminants. The response of fuel isolates to nine of the antioxidants was tested and found to be essentially identical with two notable exceptions. (The exceptions are discussed below.) The compounds received are listed in Table 2 along with the concentration used in the fuels, and the gross effect on microbial survival.

Both antioxidants and corrosion inhibitors affected microbial growth at low concentrations. In Bushnell-Haas medium as little as 1 mg per ml of additive influenced microbial growth and survival. The antioxidants were in general bactericidal; however, they killed organisms at a much slower rate than the olefins tested earlier. Lubrizol 541, a corrosion inhibitor, was bactericidal and Lubrizol 802, an antioxidant, were the most bactericidal compounds among the group tested. (See Table 3.) Tolad 244 did not kill the fuel isolates studied, and the corrosion inhibitor Unicor M supported microbial growth.

With Unicor M as the only source of carbon in Bushnell-Haas medium the number of organisms present increased from 10^8 to 10^8 cells per ml in 24 hours. (See Table 4.) It is possible that this compound is a potential source of nitrogen for organisms that oxidize fuel. However, organisms in a fuel-water system containing this additive might very well use it simultaneously with the oxidation of jet fuel or, possibly, they may use it preferentially and thereby deplete the system of an added corrosion inhibitor.

TABLE 2

MICROBIAL SURVIVAL IN THE PRESENCE OF FUEL ADDITIVES

Compound	R	%S
*Unicor M	0.10	>8815.0
*Tolad 244	0.10	<250.7
*MBX-200	0.10	≤88.7
Ethyl AN 33	0.05	<1.02
*Dupont RP2	0.05	<0.38
Lubrizol 802	0.01	<0.001
*Santolene C	0.10	<0.01
*Metal Deactivator	0.01	<0.01
*Lubrizol 541	0.05	<0.01

Legend: $R = \frac{\text{ml additive}}{\text{ml NBH}}$

%S = (Viable cell count after 24 hours/initial viable count) x 100. All viable counts were made after 24 hours exposure at 30°C.

The indicated concentrations of additive were added to Bushnell-Haas medium. Each medium was inoculated with 5 mls of culture 101 grown on fuel. The cells had been washed 3 times in distilled water. Viable counts were made in TGY agar at periodic intervals for 24 hours. Incubation: 30°C.

*Products qualified under military specification MIL-1-25017 (QPL-25017-7, 26 August 1963).

TABLE 3

EFFECT OF ANTIOXIDANT LUBRIZOL 802 ON GROWTH OF CULTURE 101

ml of additive per 100 ml NBH	0 hr	% Survival 0 hr	2 hrs	% Survival 2 hrs	24 hrs	% Survival 24 hrs
0 ml	1.40×10^{7}	100%	6.2×10^{6}	44.28%	1.62×10^{7}	115.71%
0.05 ml	1.29×10^{7}	100%	1.65×10^{5}	1.27%	no growth	0.00%
0.1 ml	3.1×10^{6}*	23.84%	2.8×10^{5}	2.15%	no growth	0.00%
1.0 ml	5.7×10^{5}*	4.39%	3×10^{2}	0.0023%	no growth	0.00%
5.0 ml	3×10^{1}*	0.00023%	no growth	0.00%	no growth	0.00%
10.0 ml	no growth	0.00%	no growth	0.00%	no growth	0.00%

* inoculated with 1.3×10^{7} cells.

Legend: See Table 2.

TABLE 4

EFFECT OF CORROSION INHIBITOR UNICOR "M" ON GROWTH OF CULTURE 101

ml of additive per 100 ml NBH	0 hr	% Survival 0 hr	2 hr	% Survival 2 hr	4 hr	% Survival 4 hr	6 hr	% Survival 6 hr	24 hr	% Survival 24 hr
0 ml	1.37×10^7	100%	9.7×10^6	70.80%	1.10×10^7	80.20%	1.26×10^7	91.97%	1.99×10^7	145.25%
0.05 ml	1.29×10^7	100%	$133. \times 10^7$	103.10%	1.34×10^7	103.87%	1.17×10^7	90.69%	2.51×10^7	194.57%
0.1 ml	9.6×10^6	100%	9.9×10^6	103.12%	1.09×10^7	113.54%	1.34×10^7	139.58%	2.31×10^7	240.62%
1.0 ml	9.3×10^6	100%	9.1×10^6	97.84%	1.13×10^7	121.50%	1.27×10^7	136.55%	7.2×10^7	774.19%
5.0 ml	8.0×10^6	100%	9.9×10^6	123.75%	8.7×10^6	108.75%	1.36×10^7	170.00%	5.1×10^8	6375%
10.0 ml	7.6×10^6	100%	1.29×10^7	169.73%	1.30×10^7	171.05%	9.0×10^6	118.42%	6.7×10^8	8815.78%

Legends: See Table 2.

2. Effects on Corrosion of Aluminum Alloys

The ability of fuel additives to prevent corrosion caused by biologically essential ions was tested in solutions containing $CaCl_2$, $FeCl_3$, or NaCl all at 8×10^{-4} moles at pH 7. Approximately 1.0 mg of additive per ml of test solution was used, and tests were made of the corrosion of the alloys 2024 and 7075. Lubrizol 541 and TRI 182 inhibited corrosion as well as 10^{-3} moles of nitrate ion did. If it is assumed that these compounds have a molecular weight of about 300 then they are as effective as corrosion inhibitors as nitrate. The other corrosion inhibitors tested were almost as good as nitrate.

The effect of antioxidants on aluminum corrosion caused by iron, calcium, and chloride compounds was also examined. These compounds did not inhibit corrosion but the character of the corrosion produced in their presence was decidedly different from that observed with the elements alone. The antioxidants, which are themselves bactericidal, appear to concentrate at the surface of these aluminum alloys. When this happens the alloy turns red and the odor of the fuel additive is detectable on the surface.

Future studies will be made of the effect of these additives on the formation of emulsions and sludges by microorganisms and on their ability to oxidize hydrocarbons and other substrates. Such additives are normal consitituents of jet fuels and their contribution to the problem of microbial fuel contamination should be assessed.

C. Chemical Characteristics of Microbial Products

During the course of this research two types of water-insoluble contaminants formed by microorganisms have been observed; one contaminant has the appearance of an emulsion and consists of fuel-oxidizing microorganisms having a specific gravity less than water. These organisms float on water and in a culture medium composed of an aqueous phase with jet fuel overlay, they are pushed into the fuel layer with agitations of the fuel-water system. The other contaminant has been called microbial sludge and it was produced in very old cultures when nitrate was the only source of nitrogen. This sludge is heavier than water and accumulates on the bottom of flasks containing such cultures.

1. Chemical Analysis of Floating Cells

Organisms growing in fuel-water systems may, or may not, form emulsions, depending on the ionic content of the growth medium, the phase or age of the culture, and the species of the organisms considered. Of these variables the age of the culture appears to be of greatest importance. In the course of this study, organisms have been isolated which cause emulsion formation in 24 hours at populations of approximately 10^8 organisms per ml, while other organisms form microbial emulsions at these cell populations after 48 hours or 72 hours of growth. The dependence of emulsion formation

on growth conditions suggests that the production of cells that float in water and cause emulsions is an enzymatically-controlled reaction, which may cause the slow accumulation of some metabolic product of low specific gravity.

A product with low specific gravity appears to be held within the cell wall or to diffuse from the cell very slowly. In this period, the lipid content of cells which form emulsions and also float were compared with those having a specific gravity greater than water; i.e., those which are dispersed through the growth medium.

The organisms which cause emulsion in a fuel-water system are referred to as top cells; the other water-dispersed organisms are called bottom cells. The analysis of these cells for lipid content was accomplished by taking weighed samples and extracting with acetone in a micro-Soxphlet apparatus for 48 hours.

Portions of the extracts were analyzed by thin-layer chromatography. A 1/2-mm layer of silica gel G was applied to the plates, dried at 100°C for 1 hour, and stored at room temperature. The elution solvent was a mixture of 70% n-propanol and 30% 1 N NH_4 OH. Approximately 500 µg of each extract was applied to the plates and eluted. After spraying with 3,6'-dichlorofluorescein, the R_f values were obtained and compared with those of known standards.

Portions of the bacterial extracts were esterified with 10% HCl in methanol at 100°C for 3 hours. After cooling, excess dimethoxypropane was added and the lipids were evaporated to dryness. The resulting fatty acid methyl esters were analyzed in a Beckman GC-2 hydrogen-flame detector gas chromatograph. Table 5 shows the gross lipid content of water-bottom cells.

The lipid content of top-layer and bottom-layer cells appeared to be essentially the same with respect to the general type of lipid present. The top layer cells, however, had about 6 times more lipid than the bottom-layer cells. The phenomenon of flotation was associated with the production of large quantities of lipids which were normal to these fuel isolates. The emergence of top layer emulsion-forming cells seemed to result from the utilization of metabolic pathways common to both types of organisms.

2. Sludge Formation by Oxidation of Hydrocarbons by Fuel Isolates and the Chemical Composition of Sludge

When fuel isolates oxidize JP-4 fuel on a medium containing, as the only source of nitrogen, comparatively high concentrations of nitrate, two compounds are produced which have not been observed so far with growth at low nitrate concentrations, or growth on substrates other than jet fuel. One of these compounds has been shown to be fuel-soluble at hydrogen ionic concentrations that permit microbial growth. The other compound is fuel-insoluble and is also insoluble in water at least from pH 3 to pH 11. These materials were studied for two reasons; first they are potential fuel contaminants, and second they should give some insight into the metabolic function of fuel organisms.

TABLE 5

A COMPARISON OF LIPID CONTENT OF TOP AND BOTTOM CELLS

	Top Layer	Bottom Layer
Weight of Bacteria	60.5 mg	269.1 mg
Weight of Extract	20.7 mg	14.6 mg
% Lipid Extract	34.0%	5.4%
Type of Lipids	Equal amounts of phospholipids, monoglycerides, triglycerides	Equal amounts of phospholipids, monoglycerides triglycerides

In the analysis, accomplished during this period, an attempt was made to acetylate the microbial sludge by refluxing with acetic anhydride. This treatment resulted in the partial dissolution of the sludge in the acetylating agent. The dissolution of this material may have resulted from the masking of charged groups by acetylation.

The acetylated material, or that material treated with acetic anhydride, was dried and dissolved in methanol with BF_3 as a catalyst. The mixture was refluxed to permit the formation of methyl esters. Following this treatment the sludge residue in methanol-BF_3 was dissolved in benzene-ethyl ether. To this organic solution, water was added and a large quantity of brown material was extracted into the water phase with precipitate formation. The organic phase was separated and washed; it was then analyzed for long-chain methyl esters. The results of that analysis are shown in Table 6. The large proportion of long-chain acids present was of interest. The identity of the fuel components that act as substrates for the biosynthesis of C_{22} acids appears to be of particular importance because of the emulsifying properties of long-chain fatty acids. The appearance of fatty acids in this transacetylated material leaves open the status of the long-chain acids in the native bacterial sludge. If the long-chain acids contained more than one functional group they could act as ligands and concentrate mineral constituents of the medium which cause corrosion. These ligands with their bound metals would agglomerate at the bottom of fuel-water systems and exercise a corrosive effect over a confined area.

A portion of the bacterial sludge was further analyzed. It was dialyzed for 24 hours and subjected to C, H, and N analyses. (Table 7.) Another portion (0.104 g) was extracted with 10% HCl-methanol for 48 hours. The resulting material was separated into a soluble fraction and an insoluble fraction by centrifugation. CHN analyses were performed on these fractions. The weight of the methanol-insoluble fraction actually was greater than indicated because much of it formed a residue on the centrifuge tubes. This residue was not dialyzed out because the residual material in the dialyzed sample was much greater than that present in the extracted sample. The C, H, N, and O values were adjusted to eliminate the effect of this residue. The methanol-insoluble portion contained much more nitrogen than the methanol-soluble portion, with the dialyzed portion having an intermediate value. This was to be expected since the methanol-soluble portion contained, primarily, the lipid portion. The protein concentration was obtained by assuming that normal proteins were responsible for the nitrogen concentration. The residual C, H, and O values were obtained by subtracting the CHO contributions of the protein from the total CHO concentrations. The oxygen value probably has a large error. These results appear to indicate that the protein lipid, and carbohydrate concentrations are approximately 50, 20, and 30% respectively. The presence of nitrogen in this water-insoluble, fuel-insoluble sludge suggests the presence of nitrated hydrocarbons, microbially produced, which contribute to the corrosivity of fuel-water bottoms.

TABLE 6

FATTY ACID CONTENT OF MICROBIAL SLUDGE FRACTIONS

Retention Time (minutes)	Peak Height (inches)	Probable Fatty Acid Ester (Chain length: Unsaturations)	Percent of Total Material Analyzed
13.34	0.47	14:1	0.09
13.86	0.80	14:0	0.16
14.42	0.25		0.05
17.84	16.88	16.0	3.48
18.98	14.00		2.89
19.44	20.00	17.0	4.12
21.38	2.95	18.1	0.61
21.80	1.02	18.0	0.21
23.24	6.76	19.0	1.39
26.02	0.47	21.1	0.09
26.36	1.73	21.0	0.36
28.50	419.20 (5.24 x 80)	22.0	86.45
32.74	0.38		0.08
Total height	484.91		

TABLE 7

A CARBON, HYDROGEN, NITROGEN, AND OXYGEN ANALYSIS OF MICROBIAL SLUDGE

	Methanol Soluble	Methanol Insoluble	Dialyzed
Dry weight	0.0322 g	0.0187 g	0.060 g
% C		45.7	25.9
% H		7.8	6.5
% N	4.8	11.3	4.7
% Residue		4.4	39.4
Adj. C		48.0	42.8
H		8.2	10.7
N	4.8	11.9	7.8
O		31.9	38.8
Protein	30.0	74.4	48.8
Residual C		9.4	17.4
H		3.4	7.5
O		15.8	28.0

D. Metabolic Activity and Viability of Fuel Isolates in Media Containing Purified Hydrocarbons

1. The Growth and Viability of Fuel Isolates in Media Containing Purified Hydrocarbons

Many microorganisms which are capable of oxidizing fuel are capable of oxidizing the five-carbon hydrocarbon pentane. The organisms isolated from JP-4 fuel in this study, however, were unable to oxidize pentane. In an effort to characterize these organisms and their metabolic products it was necessary to determine their capacity to grow on hydrocarbons of different chain length and structure.

Hydrocarbons affect these fuel isolates in one of three ways: (1) viability is not altered and the organism does not grow, (2) the organisms are killed, or (3) the hydrocarbon supports growth. Table 8 summarizes these effects in terms of the particular alkanes and olefins studied.

Pentane, hexane, and heptane do not support growth and do not kill the fuel isolates tested. These organisms are not killed by 1-pentene and are killed only slowly by 2-pentene. However, 1- or 2-hexene or heptene, 1- or 2-octene or 1-nonene kill these fuel isolates readily. But the effectiveness of these short chain olefins end with nonene, and either 1-decene or 1-dodecene support growth to about the same extent as octane, nonane, decane, and dodecane. Population densities change on these saturated hydrocarbons from 10^6 cells per ml to about 10^8 cells per ml in 48 hours.

The toxicity of short-chain olefins is not confined to organisms that grow on fuel. Figure 6 shows that E. coli is killed more rapidly by 1-heptene than by jet fuel. E. coli is distinguished from the pseudomonads isolated from fuel by its sensitivity to jet fuel, but both organisms are sensitive to the lethal properties of 1-heptene.

2. The Respiration of Fuel Isolates on Five to Ten Carbon Alkanes and Alkenes

The failure of fuel isolates to grow on short-chain alkanes and olefins prompted a study of the effects of these short-chain hydrocarbons on the metabolic pathways essential to the life of the organism.

As shown previously, octane and nonane are readily oxidized by fuel isolates and they also support microbial growth, but octene and nonene, like hexene and heptene, are inhibitory to the oxidation of jet fuel and glucose. The response to these olefins is in sharp contrast to that of compounds containing one additional carbon atom, 1-decene. This unsaturated compound is capable of supporting growth and it is inhibitory neither to jet fuel oxidation nor to glucose oxidation. The same pattern of response is observed with 1-dodecene which supports growth and does not cause respiratory inhibition.

TABLE 8

THE GROWTH AND VIABILITY OF FUEL ISOLATES IN MEDIA CONTAINING PURIFIED HYDROCARBONS

Hydrocarbon	Viable Response
Pentane	No Growth
1-Pentene	No Growth
2-Pentene	Kills
Hexane	No Growth
1-Hexene	Kills
2-Hexene	Kills
Heptane	No Growth
1-Heptene	Kills
2-Heptene	Kills
Octane	Growth
1-Octene	Kills
2-Octene	Kills
Nonane	Growth
1-Nonene	Kills
Decane	Growth
1-Decene	Growth
Dodecane	Growth
1-Dedecene	Growth

Legend: The organism tested was the fuel isolate Culture 101. These cells were grown on BH medium with a fuel overlay. They were harvested by washing 3 times in water and the washed cells were used as inocula in media overlayed with the purified hydrocarbons shown above.

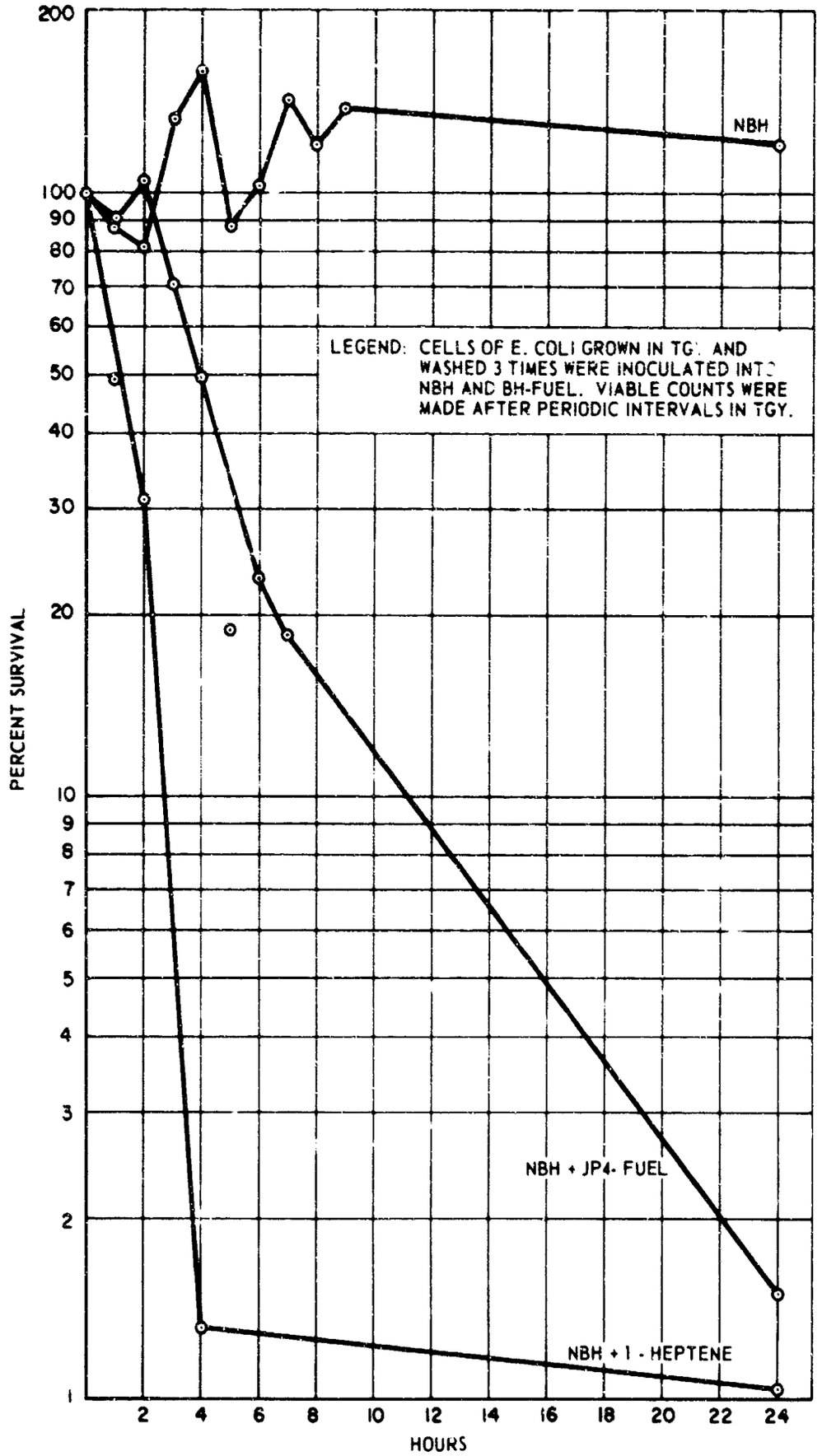


Figure 6. Effect of 1-Heptene on the Viability of E. Coli.

Table 9 summarizes our experience with short-chain hydrocarbons and their ability to undergo oxidation or affect respiratory inhibition. A knowledge of the site of action of the unsaturated hydrocarbons, which inhibit, should be of considerable importance in understanding the physiological mechanisms by which microorganisms oxidize fuel and contribute to the formation of microbial sludges and emulsions.

E. The Growth of Microorganisms in Media Containing Sealants and Topcoats

1. Microbial Deterioration of Coatings

The objective of this work was to determine whether coatings used for coating panels would support the growth of fuel microorganisms and whether corrosion would be caused by microbial removal of coating.

A number of top coatings and sealants are mentioned in this report. Generic names, the type product, and the source of the coating may be found in the Fourth Quarterly Report.[3]

a. Visual Inspection of Coated Panels: Final readings on various coatings are recorded in Table 10.

The inspection of the coating panels was quite detailed. Each panel was removed from the fuel-water medium, dried, and inspected for obvious film failure. The panels were then scanned at 20X to determine small holidays and flaws. Finally, an attempt was made physically to strip the coating from the substrate and, thus, determine the degree of adherence. Where it was possible to strip the coating cleanly, the underlying metal was examined for corrosion associated with flaws in the coating.

The majority of "sterile" controls now contain microorganisms. These were quite possibly introduced with the nonsterile coatings or during the previous inspection. In most cases, the growth in the control is much less than in the inoculated series. Sterile controls were maintained with Furan 200 and CS-3600.

b. Swelling: Swelling has nearly always been noted in Buna-N type coatings as well as in other types of polymers. Careful microscopic examination of swelled coatings indicates a very sharp line of demarcation. The coatings appear to lose adherence under these circumstances, but little damage is noted visually. The microscopic examination indicates that swelling may be symptomatic of more severe coating damage.

c. Pitting under Holidays: It has been often stated that corrosion will occur under holidays* in coatings. Observations in the field have substantiated the occurrence of pits under flaws in coatings; but, to our

* Holiday -- pinhole; skip; discontuity; void.

TABLE 9

THE RESPIRATION OF FUEL ISOLATES IN THE PRESENCE OF SHORT-CHAIN SATURATED AND UNSATURATED HYDROCARBONS

Hydrocarbon	Oxygen Uptake	Effect on Fuel Oxidation	Effect on Glucose Oxidation
Pentane	No	Slight Inhibition	Slight Inhibition
1-Pentene	No	Inhibition	Inhibition
2-Pentene	No	Inhibition	Inhibition
Hexane	Variable: Always Small	Slight Inhibition	Slight Inhibition
1-Hexene	No	Inhibition	Inhibition
2-Hexene	Variable: Always Small	Inhibition	Inhibition
Heptane	Not Sustained	No Inhibition	No Inhibition
1-Heptene	Not Sustained	Inhibition	Inhibition
2-Heptene	No	Variable, Inhibited	Inhibition
Octane	Yes	No Inhibition	No Inhibition
1-Octene	No	Inhibition	Inhibition
Nonane	Yes	No Inhibition	No Inhibition
1-Nonene	No	Inhibition	Inhibition
Decane	Yes	No Inhibition	No Inhibition
1-Decene	Yes	No Inhibition	No Inhibition
Dodecane	Yes	No Inhibition	No Inhibition
1-Dodecene	Yes	No Inhibition	No Inhibition

TABLE 10

OBSERVATION OF COATED PANELS

Coating	Panel	Color Extraction	Time of Incubation (days)	Comment
Polysulfide 890	Steel	None	135	Fading in water phase; swelling in water phase; blistering in water phase, slight edge corrosion.
Polysulfide 890	Aluminum	None	135	As above, except no edge corrosion
Buna 776	Steel	In Fuel	135	Mottled in fuel and Water phases; blistering in water phase; corrosive pits under blisters.
Buna 776	Aluminum	In Fuel	135	Leaching in fuel phase; dulled and roughened in water phase. Slight blistering in water phase and pitting.
Polyurethane 1560	Steel	None	168	No effect.
Polyurethane 1560	Aluminum	In Fuel	168	Slight fading in water phase
Furan 200	Steel	None	135	Blistering in water phase varied from very slight to heavy with all inoculated samples showing some blistering. Adherence poor.
Furan 200	Aluminum	None	135	Slight fading in water phase. Excellent adherence.

TABLE 10 (Continued)

OBSERVATION OF COATED PANELS

Coating	Panel	Color Extraction	Time of Incubation (days)	Comment
3C-3001	Steel	In Fuel	130	Leaching in fuel, fading and blistering in water phase.
3C-3001	Aluminum	In Fuel	130	Leaching in fuel phase; bleaching in water phase. Large blisters with bubbles in the vapor phase and pinholes in the liquid phase.
CS-3600	Steel	In Fuel	130	Leaching in fuel phase; mottling in water phase.
CS-3600	Aluminum	In Fuel	130	Leaching in fuel phase; mottling in water phase.
Zincilate 101	Steel	None	130	Topcoat blistered and peeling in fuel phase; peeling in air on one sample; edge failure of primer.
Zincilate 101	Aluminum	None	130	Peeling of topcoat in water phase.
Polyurethane 2610	Steel	None	130	Very slight discoloration in the water phase.
Polyurethane 2610	Aluminum	None	130	Very slight discoloration in the water phase.

TABLE 10 (Continued)

OBSERVATION OF COATED PANELS

Coating	Panel	Color Extraction	Time of Incubation (days)	Comment
833-5	Steel	In Fuel	130	Bleaching and increased blistering in the water phase; leaching in fuel phase.
833-5	Aluminum	In Fuel	130	As above but with less blistering.
Polysulfide 1422	None (filets)	None	130	Faded in the water phase

* NOTE: Growth medium to heavy on all inoculated samples. Controls were contaminated except in the case of furan 200 and CS-3600. Trace to very light growth was present in the controls on Buna 776 and Polyurethane 2610. See text.

knowledge, this has rarely been documented in the laboratory. Figure 7 clearly demonstrates a holiday and the corrosion immediately under it. These photographs are of Buna-N and aluminum.

Heavy corrosion was observed under coatings that were severly blistered. Figure 8 is a photomacrograph of a blistered panel which demonstrates the corroded areas under the blisters.

The formation of small bubbles in top coatings is very common and considered undesirable. However, little attention has been paid to them if the bubbles were small in size and few in number. Our observations indicate that bubbling may be a more severe problem than generally recognized. In many cases, the bubbles apparently break down and form pinholes in the liquid phase. Little or no effect was noted in the vapor phase. Figure 7 also shows one of the very small pinholes in a Buna-N coating.

2. Effect of Soil Burial on Coatings

As described in the Third Quarterly Report, one of the techniques planned was to obtain adapted cultures from coatings subjected to soil burial in the presence of JP-4. The work on coatings was terminated before isolations of the microflora were made but the following observations were made and thought to be of interest.

Excellent mold growth has been obtained on the soil-manure-water-JP-4 mixture. The fungi are mixed but large numbers of an actinomyces grew along with numbers of Alternaria and, probably, Trichothecium.

Empirically, it appeared that the Buna-N type and furan-type coatings may have lost plasticizer, since they are quite brittle. The polysulfide and polyurethane do not seem to have been affected.

Fungal growth has been demonstrated on both the furan and the Buna-N coatings. The coatings were removed, washed to remove adhering soil, and examined. Figure 9 illustrates fungal hypha on the furan and Figure 10 shows fungal mycelia on the Buna-N. It is not known whether the hypha penetrated the coating. However, the microscopic appearance is very similar to fungal growth on other molded coatings.

a. Inspection of Hazzard-Type Tests: Nylon net was coated with various polymers under investigation, using a modification of the procedure recommended by Hazzard and reported in the Third Quarterly Report. The results of the series has confirmed the other data reported here. Failure, as demonstrated by growth, was obtained with Buna-N, furan, and zincilate. The polyurethane and polysulfide coatings were not penetrated by microorganisms. Work was discontinued on the Hazzard tests since it was difficult to obtain satisfactory coating films.

Figure 7. Photomacrograph of Coated Coupon with Coating Pealed Back to Show the Holiday and Corresponding Pit in the Metal

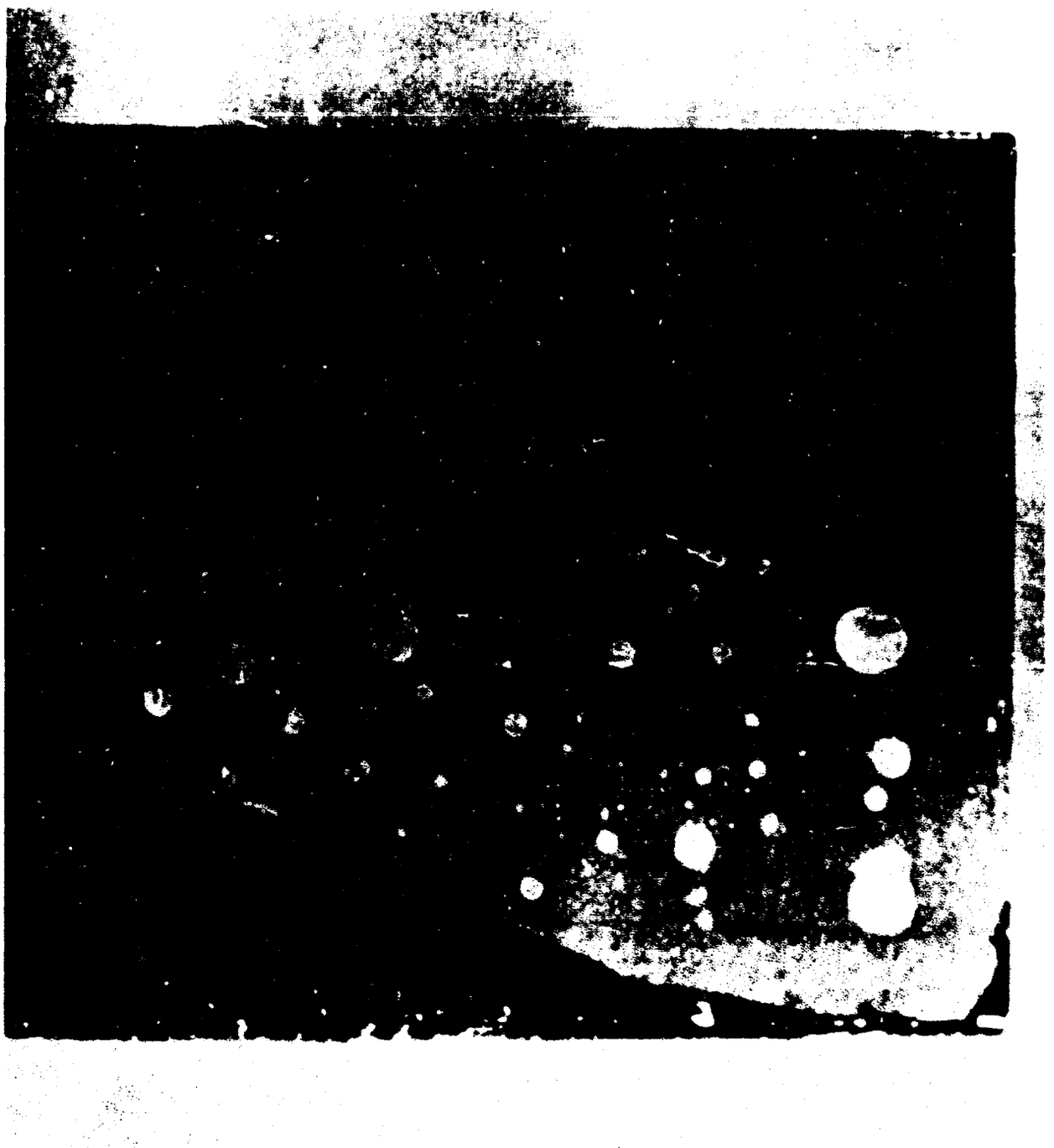


Figure 8. Photomacrograph of Coated Coupon with Severe Blistering. Blistered Coating has Been Stripped to Show Corrosion

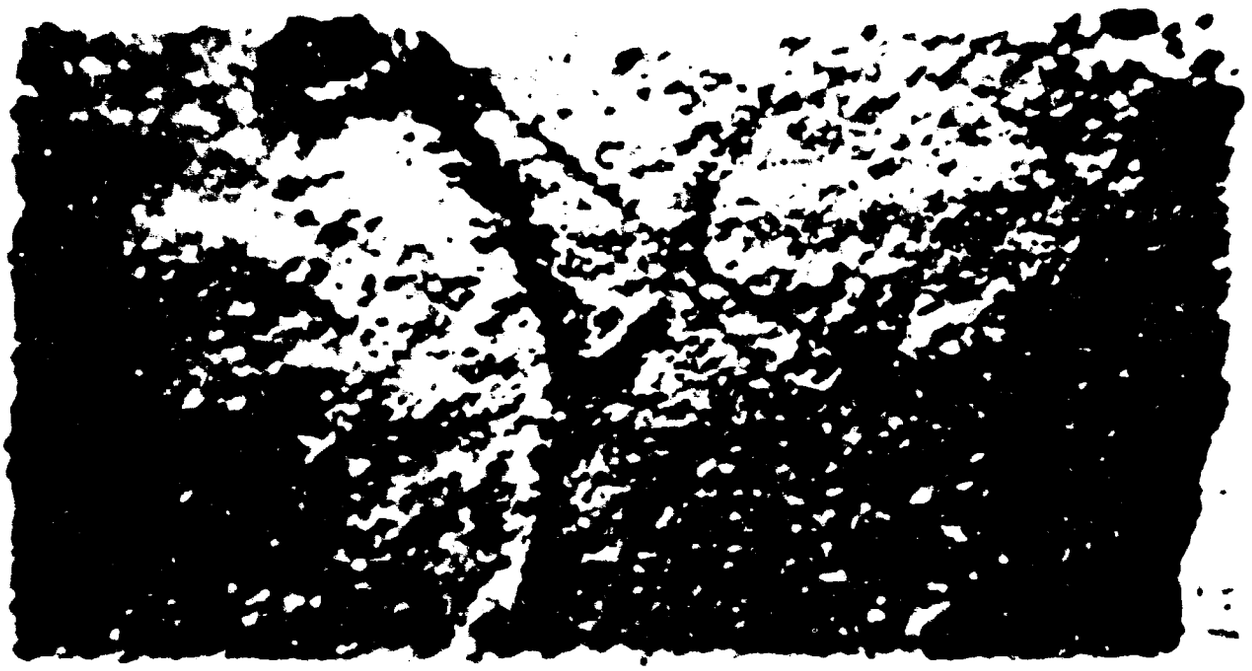


Figure 9. Photomicrograph of Fungal Hypha on Furan Type Coating (Oblique View)

Figure 10. Photomicrograph of Fungal Hypha on Buna-N Type Coating

3. Utilization by Microorganisms of Combined and Uncombined Nitrogen Contained in Aircraft Coatings

It is well known that uncombined nitrogen extracted from aircraft coatings by water will support adequately the nitrogen requirements of microorganisms. What is not known is whether microorganisms are capable of extracting from aircraft coatings that nitrogen which is combined with other atoms to form an integral part of the coating polymer. If microorganisms are capable of extracting combined nitrogen to satisfy their nitrogen requirements, the coating will deteriorate with subsequent loss of function.

In order to determine the ability of microorganisms actively to extract nitrogen from coatings, test microorganisms were grown in both extracted and nonextracted coatings. Growth was determined turbidimetrically and expressed as the percent transmittance at 525 mμ.

The medium used in these experiments did not contain nitrogen. However, previous reports have demonstrated that sufficient nitrogen is present in the form of trace contaminants to permit good growth of these undemanding microorganisms. Thus, prior to use in these experiments, the medium was exhausted of trace nitrogen by culturing with microorganisms for seven days at 28°C. Cells were removed from the medium by filtration through a 0.47 μ membrane filter.

To remove the uncombined nitrogen, Buna-N type coatings were extracted three times by boiling in distilled water. The water extracts were combined and used in the experiments. It has been previously demonstrated that the fourth water extraction from Buna-N will not support appreciable growth in exhausted media and it is assumed that the bulk of water-extractable nitrogen has been removed by the three extractions.

Several experiments were performed. Results from one of the typical series are recorded in Table 11 and indicate that some microorganisms are apparently able to remove nitrogen from washed coatings. The Bacillus subtilus culture grew better with extracted Buna-N coating than in the medium without coating. Oddly, the results on the mixed culture indicate that the mixed bacteria did not actively utilize nitrogen from the coating in this manner.

The data from the mixed culture are confusing. The low level of nutrients has apparently caused filament and slime formation, and both turbidity and direct counts are unreliable.

In summary, a number of coated aluminum and steel coupons incubated in the presence of mineral salts, JP-4, and bacteria have been examined. The data show polyurethane coatings to be quite resistant to deterioration in these tests; most of the other types of coatings have some degree of failure.

TABLE 11

COMBINED AND UNCOMBINED NITROGEN UTILIZATION BY MICROORGANISMS

Test Organism	Substrate	Percent Transmittance[a]		
		I	II	III
B. subtilus	(1) exhausted medium only	98.0	97.0	contaminated
	(2) exhausted medium plus non-extracted Buna-N	92.0	90.0	85.0
	(3) exhausted medium plus water extract	72.0	80.0	64.0
	(4) exhausted medium plus extracted Buna-N	77.0	92.0	60.0
Culture X (Mixed bacterial culture)	(1) exhausted medium only	88.5	94.0	86.0
	(2) exahusted medium plus non-extracted Buna-N	95.0	94.0	93.0
	(3) exhausted medium plus water extract	89.0	87.0	88.5
	(4) exhausted medium plus extracted Buna-N	94.0	91.0	92.0

Legend: a. Growth recorded as percent transmittance at 525 mμ after seven days incubation at 28°C.

Nitrogen-free medium:

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.4	g
Calcium chloride ($CaCl_2$)	0.02	g
Potassium phosphate, dihydrogen (KH_2PO_4)	2.0	g
Potassium phosphate, monohydrogen (K_2HPO_4)	2.0	g
Dextrose	1.0	g
Distilled water	1000	g

Pit corrosion under blisters and pinholes in coatings have been demonstrated and documented. One series of coatings have shown a correlation between blister formation and the presence of bacteria. Other phases of the test work on coatings have been reported and concluded.

The microbial utilization of bound nitrogen in coatings has been further investigated with additional evidence, but not final proof, that microorganisms remove nitrogen directly from the coatings.

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